Expression of inter- α -trypsin inhibitor heavy chains in endometrium of cyclic and pregnant gilts

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Attachment of the placenta to the uterus in pigs involves extracellular interaction between the expanding trophoblastic membrane and the thick glycocalyx present on the uterine epithelial microvilli. Formation of complexes between members of inter- α -trypsin inhibitor family may function in the maintenance of the extracellular matrix. This study investigated the change in the inter- α -trypsin inhibitor heavy chains (ITIH1, ITIH2, ITIH3 and ITIH4) during the oestrous cycle and early pregnancy in pigs. Gene expression of *ITIH1*, *ITIH2*, *ITIH3* and *ITIH4* was detected in the endometrium of cyclic and pregnant

gilts; however, gene expression of *ITIH* was not altered throughout the oestrous cycle or early pregnancy. Western blot analysis with an ITIH antiserum identified the possible linkage forms of ITIH with the serine protease inhibitor, bikunin. Pregnancy altered the release of the various inter- α -inhibitor forms from the endometrium during the period of trophoblastic attachment. The results from this study indicate that the inter- α -trypsin inhibitor family plays an important role in maintenance of the uterine surface glycocalyx during placental attachment in pigs.

Introduction

Survival of developing pig embryos within the uterus is dependent upon formation of an epitheliochorial type of placental attachment to the uterine surface. Pig conceptuses initiate attachment (days 13–18) to the thick glycocalyx present on the apical border of the surface epithelium after rapid expansion of their trophoblastic membrane on day 12 of pregnancy (King *et al.*, 1982; Strobant and Van der Lende, 1990; Geisert and Yelich, 1997). Elongation and expansion of the trophoblastic membrane of the conceptus involves changes in cellular morphology through activation of many embryonic factors (Yelich *et al.*, 1997a,b) and the presentation of uterine adhesion factors on the epithelial surface that permit continuous attachment of the placenta throughout gestation (Bowen *et al.*, 1996; Jaeger *et al.*, 2001). During adhesion of the trophoderm to the uterine epithelial apical surface, a localized increase in transcapillary transport (Keys and King, 1988) as well as an overall increase in uterine blood flow occurs (Ford and Christenson, 1979) concurrently with conceptus synthesis of oestrogen (Ford *et al.*, 1982; Geisert *et al.*, 1982) needed for maintenance of the corpora lutea and establishment of pregnancy in pigs.

Many of the endometrial responses evoked by the developing pig conceptuses during this period of early development and uterine attachment resemble the acute phase response induced during generalized tissue inflammation (Salier *et al.*, 1996; Geisert and Yelich, 1997). A glycoprotein member of the acute phase proteins, inter- α -trypsin inhibitor heavy chain 4 (ITIH4), has been isolated and characterized in the endometrium of pigs (Geisert *et al.*, 1998). ITIH4 is only one member of the inter- α -trypsin inhibitor family of protease inhibitors. The inter- α -trypsin inhibitor family is composed of four heavy chains (ITIH1, ITIH2, ITIH3 and ITIH4) and one light chain, bikunin (Salier *et al.*, 1996). Linkage of the various family members is proposed to be involved with stabilization of the extracellular matrix (Bost *et al.*, 1998). Binding of bikunin to ITIH occurs in three forms, pre- α -inhibitor (P α I), inter- α -like inhibitor (I α LI) and inter- α -inhibitor (I α I). P α I is a complex of bikunin bound to a single heavy chain of ITIH3; I α LI is composed of bikunin and ITIH2 (Enghild *et al.*, 1991); and I α I is formed by covalent binding of bikunin with two heavy chains, ITIH1 and ITIH2 (Bost *et al.*, 1998). Attachment of bikunin to the heavy chains occurs through binding to a chondroitin sulphate chain. The serine protease inhibitory activity of I α I complex originates from bikunin, a 30 kDa serine protease inhibitor containing two kunitz-type inhibitory domains (Xu *et al.*, 1998), as the heavy chains are devoid of protease inhibitory activity. ITIH4 is unique from other ITIH as the Asp-Pro-His-Phe-Ile-Ile sequence for binding

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to bikunin is absent (Enghild *et al.*, 1989; Salier *et al.*, 1996; Bost *et al.*, 1998) and, in contrast to the other ITIH, is a substrate for the plasma serine protease kallikrein (Nishimura *et al.*, 1995).

As ITIH function as hyaluronic binding proteins, it has been proposed that $\alpha\alpha$ I, $\alpha\alpha$ LI and $\alpha\alpha$ L stabilize the extracellular matrix (Bost *et al.*, 1998). Evidence for a role of the ITI family in expansion of the cumulus–oocyte complex (Chen *et al.*, 1994, 1996) supports a principal function of the ITI family in matrix stabilization through interaction with hyaluronic acid. The non-invasive, superficial epitheliochorial placentation in pigs is associated with a reduction in the uterine epithelial glycocalyx between the interdigitating trophoblastic and uterine epithelial microvilli (Dantzer, 1985). Although there is limited information concerning the apical interactions between the trophoblast and uterine surface epithelium, results indicate that changes in the cellular apical glycocalyx play a major role in initial trophoblastic attachment (Bowen *et al.*, 1996; Jaeger *et al.*, 2001). Detection of ITIH4 (Geisert *et al.*, 1998) and bikunin (Hettinger *et al.*, 2001) in the endometrium of pigs indicate that the $\alpha\alpha$ I family may be involved with maintenance of the uterine apical glycocalyx necessary for trophoblastic attachment in pigs (Stroband and Van der Lende, 1990). The aim of the present study was to evaluate endometrial gene expression of *ITIH1*, *ITIH2*, *ITIH3* and *ITIH4* and the presence of $\alpha\alpha$ I, $\alpha\alpha$ L and $\alpha\alpha$ LI in the uterus during the oestrous cycle and early pregnancy of pigs.

Materials and Methods

Animals

Research was conducted in accordance with the Guiding Principles for Care and Use of Animals promoted by the Society for the Study of Reproduction and approved by the Oklahoma State Institutional Animal Care and Use Committee. Cyclic, large white gilts of similar age (8–10 months) and weight (100–130 kg) were examined twice each day for oestrous behaviour using intact boars. The onset of oestrus was considered as day 0 of the oestrous cycle. Gilts that were to be mated were bred naturally with fertile boars at the first detection of oestrus and 12 h later.

Cyclic gilts ($n=18$) were hysterectomized on days 0, 5, 10, 12, 15 or 18 of the oestrous cycle, whereas pregnant gilts ($n=12$) were hysterectomized on days 10, 12, 15 or 18 of gestation as described by Gries *et al.* (1989). After surgical removal of the uterine horns as described by Gries *et al.* (1989), uterine flushings were obtained by isolating one uterine horn and flushing with 20 ml of PBS (pH 7.4). Uterine flushings were placed on ice until centrifugation (2500 *g*, 10 min, 4°C). After flushing, the uterine horn was cut along its antimesometrial border; the endometrium was collected, snap frozen in liquid nitrogen and stored at -80°C . The remaining uterine horn was immediately placed in a sterile container and

transported on ice for explant culture. Endometrium was removed from the mesometrial side of the uterine horn and cut into 4 mm $\times$ 4 mm sections. A total of 0.5 g explant tissue was placed in 15 ml Dulbecco's modified Eagle's medium (DMEM) (Gibco/Life Sciences, Gaithersburg, MD) and 2% (v/v) antibiotic–antimycotic (Gibco/Life Sciences). After an initial incubation for 3 h, medium was replaced with fresh medium to remove serum leaching from the tissue. Endometrial explant cultures were incubated in air on a rocking platform (four cycles per min) for an additional 24 h in DMEM at 37°C. Endometrial explant culture medium was centrifuged at 2500 *g* for 10 min at 4°C. Endometrial tissue, uterine flushings and endometrial explant medium were stored at -80°C until analysed.

Western blot analysis

Uterine flushings and endometrial explant culture media were analysed by western blot analysis, as described by Geisert *et al.* (1995). Polypeptides in uterine flushings and explant culture medium (50 μg total protein) were separated by 12.5% one-dimensional SDS-PAGE and immediately transferred to polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA). After electroblotting, the membranes were washed, blocked and incubated with a 1:750 dilution of sheep first antibody raised against human inter- α -trypsin inhibitor (The Binding Site, Inc., San Diego, CA). Sheep non-immunized serum was run as a negative control. Immunoreactive polypeptides were detected using the Bio-Rad Immuno-Blot kit (Bio-Rad, Hercules, CA) according to the manufacturer's specifications.

Endometrial RNA extraction

Total RNA was isolated from endometrial tissue using TRIzol reagent (Gibco/Life Sciences). Approximately 0.5 g of endometrial tissue was homogenized in 5 ml TRIzol reagent using a Virtishear homogenizer (Virtis Co. Inc., Gardiner, NY). RNA pellets were rehydrated with 10 mmol Tris l^{-1} , 1 mmol EDTA l^{-1} (pH 7.4) and stored at -80°C until further analysis. Total RNA was quantified using a spectrophotometer at an absorbance of 260 nm, whereas the purity was determined based on 260:280 nm ratios. Integrity of the RNA was examined via gel electrophoresis.

Total RNA was reverse transcribed to cDNA using Moloney murine leukaemia virus reverse transcriptase (Promega Corporation, Madison, WI). The quality of endometrial cDNA was verified by amplifying glyceraldehyde-3-phosphate dehydrogenase as described by Yelich *et al.* (1997a).

ITIH1, *ITIH2* and *ITIH3* primer construction, optimization and sequencing

ITIH1, *ITIH2* and *ITIH3* primers were designed from pig cDNA sequences available in GenBank to determine

Table 1. PCR primer and probe sequences used for quantitative RT-PCR

Gene		Forward primer/reverse primer/probe	GenBank accession number
<i>ITIH1</i>	Forward	5'-TGA CTGGCGTGGACACTGA-3'	Y11546
	Reverse	5'-GCACAGGGTGCCTCTTTCTG-3'	
	Probe	5'-CCCCACTTCATCATCCGCGTGC-3'	
<i>ITIH2</i>	Forward	5'-CGCGGTAGGACCTTCAGTGA-3'	Y11545
	Reverse	5'-GAAGTGCGGGTCATTTTCGA-3'	
	Probe	5'-AGGTAATGCCTCCTCCACACGTGATGA-3'	
<i>ITIH3</i>	Forward	5'-TGGTTTGTTACGACAATGG-3'	D84320
	Reverse	5'-AGGACGACTCTGTCGATCGG-3'	
	Probe	5'-TGTCCTCCGACCTGTTCTGAGTGG-3'	
<i>ITIH4</i>	Forward	5'-TGCCTACAAGTGAAGGAAACA-3'	U43164
	Reverse	5'-CCCGCCTTGTCATGGT-3'	
	Probe	5'-TCTACTCAGTGATGCCCGGCCTCAA-3'	

ITIH: inter- α -trypsin inhibitor heavy chain.

whether they were expressed in the endometrium of pigs. The nucleotide sequence of pig *ITIH1* (GenBank Y11546) was used to construct the forward 5'-GCTGGA-GAACTATGTGCAACGC-3' (1276–1297 bp) and reverse 5'-AAGTAGGTGCCCTCATGCTTCC-3' (1773–1794 bp) primers. The nucleotide sequence of pig *ITIH2* (GenBank Y11545) was used to construct forward 5'-AGTCTTGCT-CAGGTACCC-3' (1948–1969 bp) and reverse 5'-TCGA-CATTGATCGGATGCTTC-3' (2504–2524 bp) primers. The forward 5'-TGGTTTGTTACGACAATGG-3' and reverse 5'-TCACCTTGCCCTTTATTATGC-3' primers for *ITIH3* were designed from partial sequences of pig liver *ITIH3* mRNA (GenBank D84319). The PCR conditions were optimized by pooling cDNA from cyclic and pregnant endometrium of all days and amplifying with 0.6 U Taq DNA polymerase and its supplied MgCl_2 -free buffer (Promega, Madison, WI) and a $3 \times 2 \times 3$ factorial combination of primer (50, 150, 250 nmol l^{-1}), deoxy-nucleotide triphosphates (dNTPs; 50 or 100 $\mu\text{mol l}^{-1}$), and MgCl_2 (1.25, 2.50 or 3.75 mmol l^{-1}) as described by Yelich *et al.* (1997a). Conditions used for *ITIH1*, *ITIH2* and *ITIH3* gene amplification were 5 nmol l^{-1} of template in 1.25 mmol $\text{MgCl}_2 \text{l}^{-1}$, 100 $\mu\text{mol dNTPs l}^{-1}$ and 50 nmol primer l^{-1} . The Recombinant DNA/Protein Research Facility at Oklahoma State University sequenced amplicons from the PCR endometrial cDNA.

Quantitative reverse transcriptase–polymerase chain reaction

Endometrial gene expressions for *ITIH1*, *ITIH2*, *ITIH3* and *ITIH4* were quantified by using the one-step RT-PCR reaction following the manufacturer's recommendations for TaqMan[®] Gold RT-PCR kit (P/N N808-0233) (PE Applied Biosystems, Foster City, CA), as described by Hettinger *et al.* (2001). Information concerning cDNA location and the sequence of the primers and probe for

ITIH1, *ITIH2*, *ITIH3* and *ITIH4* are presented (Table 1). The total reaction volume of 50 μl contained 200 nmol forward primer l^{-1} , 200 nmol reverse primer l^{-1} , 100 nmol fluorescent labelled probe l^{-1} and 100 ng of total RNA. The PCR amplification was carried out in the ABI PRISM[®] 7700 sequence detection system (Applied Biosystems). Thermal cycling conditions were 50°C for 2 min, 95°C for 10 min followed by repetitive cycles of 95°C for 15 s and 60°C for 1 min. Ribosomal 18S RNA (18S, RNA Control Kit, 43108993E, Applied Biosystems) was run as a control for RNA loading. Reverse transcriptase was omitted from sample PCR wells to determine possible contribution of sample genomic DNA contamination to amplification. Amplification of curve of genomic DNA was similar to the no template control.

After RT-PCR, gene amplification was quantified by setting the threshold cycle (C_T) in the geometric region of the plot after examining the semi-log view of the amplification plot. Relative quantification of *ITIH* gene expression was evaluated using the comparative C_T method, as described by Hettinger *et al.* (2001). The ΔC_T value is determined by subtracting the *ITIH* C_T of each sample from its ribosomal 18S C_T value (Table 2). Calculation of $\Delta\Delta C_T$ involves using the highest sample ΔC_T value as an arbitrary constant to subtract from all other ΔC_T sample values. Fold changes in gene expression of *ITIH* is determined by evaluating the expression, $2^{-\Delta\Delta C_T}$.

Statistical analysis

Data were analysed by least-squares ANOVA using the General Linear Models of the Statistical Analysis System (SAS, 1988). The statistical model used to analyse endometrial expression of *ITIH* genes included the effects of day, reproductive status (cyclic and pregnant), and day $\times$ reproductive status.

Table 2. Comparison of endometrial expression of inter- α -trypsin inhibitor heavy chain (ITIH1, ITIH2, ITIH3 and ITIH4) genes across days of the oestrous cycle and early pregnancy using the comparative C_T method

Target	Status	Day	Average target C_T^a	Average 18S rRNA C_T^a	$\Delta C_T^{b,d}$	$\Delta \Delta C_T^c$	Fold change
ITIH1	Cyclic	0	26.81 $\pm$ 1.74	13.54 $\pm$ 0.19	13.27 $\pm$ 1.67	-0.91	1.88
	Cyclic	5	27.70 $\pm$ 2.10	13.52 $\pm$ 0.27	14.18 $\pm$ 2.00	0.00	1.00
	Cyclic	10	26.81 $\pm$ 1.10	13.24 $\pm$ 0.31	13.57 $\pm$ 1.22	-0.61	1.53
	Pregnant	10	25.82 $\pm$ 0.59	13.20 $\pm$ 0.17	12.62 $\pm$ 0.60	-1.56	2.95
	Cyclic	12	27.36 $\pm$ 1.51	13.21 $\pm$ 0.45	14.15 $\pm$ 1.10	-0.03	1.02
	Pregnant	12	26.54 $\pm$ 0.95	13.39 $\pm$ 0.08	13.15 $\pm$ 0.89	-1.03	2.04
	Cyclic	15	26.69 $\pm$ 1.12	12.96 $\pm$ 0.11	13.73 $\pm$ 1.22	-0.45	1.37
	Pregnant	15	26.91 $\pm$ 0.70	13.59 $\pm$ 0.09	13.32 $\pm$ 0.77	-0.83	1.78
	Cyclic	18	26.69 $\pm$ 2.24	12.96 $\pm$ 0.15	13.73 $\pm$ 1.73	-0.42	1.34
ITIH2	Pregnant	18	26.92 $\pm$ 1.14	13.58 $\pm$ 0.10	13.34 $\pm$ 0.89	-0.84	1.79
	Cyclic	0	30.84 $\pm$ 1.94	13.54 $\pm$ 0.19	17.30 $\pm$ 2.09	-2.30	4.92
	Cyclic	5	33.12 $\pm$ 2.14	13.52 $\pm$ 0.27	19.60 $\pm$ 1.90	0.00	1.00
	Cyclic	10	31.31 $\pm$ 1.05	13.24 $\pm$ 0.31	18.07 $\pm$ 1.07	-1.53	2.89
	Pregnant	10	32.08 $\pm$ 1.05	13.20 $\pm$ 0.17	18.88 $\pm$ 0.89	-0.72	1.65
	Cyclic	12	32.17 $\pm$ 1.43	13.21 $\pm$ 0.45	18.96 $\pm$ 1.27	-0.64	1.56
	Pregnant	12	29.71 $\pm$ 0.92	13.39 $\pm$ 0.08	16.32 $\pm$ 0.83	-3.28	9.71
	Cyclic	15	31.33 $\pm$ 1.32	12.96 $\pm$ 0.11	18.37 $\pm$ 1.35	-1.23	2.35
	Pregnant	15	31.06 $\pm$ 0.47	13.59 $\pm$ 0.09	17.47 $\pm$ 0.49	-2.13	4.38
ITIH3	Cyclic	18	30.95 $\pm$ 0.93	12.96 $\pm$ 0.15	17.99 $\pm$ 0.57	-1.61	3.05
	Pregnant	18	31.52 $\pm$ 0.58	13.58 $\pm$ 0.10	17.94 $\pm$ 0.60	-1.66	3.16
	Cyclic	0	26.97 $\pm$ 1.08	13.54 $\pm$ 0.19	13.42 $\pm$ 0.93	-0.52	1.43
	Cyclic	5	26.25 $\pm$ 0.81	12.96 $\pm$ 0.12	13.29 $\pm$ 0.81	-0.65	1.57
	Cyclic	10	26.68 $\pm$ 0.53	13.65 $\pm$ 0.07	13.03 $\pm$ 0.50	-0.91	1.88
	Pregnant	10	26.92 $\pm$ 0.60	12.97 $\pm$ 0.04	13.94 $\pm$ 0.55	0.00	1.00
	Cyclic	12	26.56 $\pm$ 0.43	13.36 $\pm$ 0.24	13.21 $\pm$ 0.41	-0.73	1.66
	Pregnant	12	25.69 $\pm$ 0.17	13.31 $\pm$ 0.15	12.37 $\pm$ 0.21	-0.57	1.48
	Cyclic	15	27.32 $\pm$ 0.56	13.51 $\pm$ 0.21	13.81 $\pm$ 0.36	-0.13	1.09
ITIH4	Pregnant	15	26.13 $\pm$ 0.49	13.64 $\pm$ 0.20	12.42 $\pm$ 0.26	-0.52	1.43
	Cyclic	18	28.28 $\pm$ 0.35	14.93 $\pm$ 0.61	13.34 $\pm$ 0.50	-0.60	1.52
	Pregnant	18	26.74 $\pm$ 0.48	13.51 $\pm$ 0.15	13.23 $\pm$ 0.61	-0.71	1.64
	Cyclic	0	30.94 $\pm$ 0.36	13.54 $\pm$ 0.19	17.40 $\pm$ 0.36	-0.55	1.46
	Cyclic	5	30.86 $\pm$ 0.17	13.52 $\pm$ 0.27	17.34 $\pm$ 0.17	0.61	1.53
	Cyclic	10	30.80 $\pm$ 0.26	13.24 $\pm$ 0.31	17.56 $\pm$ 0.26	-0.39	1.31
	Pregnant	10	30.81 $\pm$ 0.18	13.20 $\pm$ 0.17	17.61 $\pm$ 0.19	-0.34	1.27
	Cyclic	12	29.81 $\pm$ 0.64	13.21 $\pm$ 0.45	16.60 $\pm$ 0.63	-1.35	2.55
	Pregnant	12	30.79 $\pm$ 0.33	13.39 $\pm$ 0.08	17.40 $\pm$ 0.33	-0.55	1.46
	Cyclic	15	30.44 $\pm$ 0.70	12.96 $\pm$ 0.11	17.48 $\pm$ 0.70	-0.47	1.39
	Pregnant	15	30.47 $\pm$ 0.62	13.59 $\pm$ 0.09	16.88 $\pm$ 0.62	-1.07	2.10
	Cyclic	18	30.91 $\pm$ 0.64	12.96 $\pm$ 0.15	17.95 $\pm$ 0.64	0.00	1.00
	Pregnant	18	30.28 $\pm$ 0.52	13.58 $\pm$ 0.10	16.70 $\pm$ 0.52	-1.25	2.38

^a C_T = Cyclic threshold: cycle number where amplification crosses the threshold set in the geometric portion of the amplification curve.

^b ΔC_T = ITIH - 18S ribosomal C_T : normalization of PCR cycles for the ITIH target with 18S ribosomal RNA.

^c $\Delta \Delta C_T$ = Mean ΔC_T - highest mean ΔC_T value: the mean value on day 10 of pregnancy (highest ΔC_T ; lowest gene expression for ITIH1) was used as a calibrator for ITIH1, on day 5 cycling for ITIH2 and ITIH4, for ITIH3 and for ITIH4 to set the baseline for comparing mean differences in ΔC_T values across all days.

^dComparison of day, status and status by day effects were analysed with the normalized ΔC_T values. No significant differences were detected for day, status or status by day.

Results

Western blot analysis of uterine α I

The human inter- α -trypsin inhibitor antibody recognized heavy chains of α I in pig serum (Fig. 1). Antiserum detected reactive products in pig serum that were approximately 120–140 kDa and > 200 kDa, which are

consistent with the M_r of α I forms described in human serum (Rouet *et al.*, 1992). The three serum products detected by the antiserum correspond to the putative M_r of α I, α LI and α PI. However, it is possible that the polyclonal antiserum used could also detect ITIH4 at the 120 kDa band. Antiserum detected similar bands of α I in endometrial culture medium from both cyclic and

pregnant gilts (Fig. 1a). With the exception of oestrus, the three I α I reactive products were detected throughout the oestrous cycle in cyclic gilts, but expression was greatly attenuated in pregnant gilts after day 10 of gestation.

A band I α I at >200 kDa was detected in uterine flushings on day 5 and day 10 of the oestrous cycle (Fig. 1b). However, only very faint reactive products were detected in uterine flushings of cyclic gilts at oestrus and after day 10 of the oestrous cycle. The 200 kDa band was also faint to absent in uterine flushings from pregnant gilts but in contrast to cyclic gilts lower M_r products of approximately 60–50 kDa were detected in uterine flushings on days 12, 15 and 18 of the oestrous cycle.

Quantitative RT-PCR analysis of endometrial mRNA expression of ITIH1, ITIH2, ITIH3 and ITIH4

Primers designed to ITIH1 and ITIH2 amplified an endometrial RT-PCR product that had 93 and 100% similarity to pig ITIH1 and ITIH2 cDNA sequences published in GenBank (Y11546 and Y11545). Endometrium expressed a 182 bp amplicon that had 100% similarity to pig liver ITIH3 (GenBank D84319). Geisert *et al.* (1998) established endometrial gene expression for ITIH4. Endometrial expression of mRNA for ITIH1, ITIH2, ITIH3 and ITIH4 during the oestrous cycle and early pregnancy was quantified with real-time RT-PCR. Quantitative ITIH gene expression was evaluated using the comparative C_T (threshold cycle) method (see Table 2). Ribosomal 18S RNA was used to normalize each sample for variation in RNA loading. No day, status or status by day interaction was detected for PCR cycle number (ΔC_T) with endometrial expression of ITIH1, ITIH2, ITIH3 or ITIH4 genes (Table 2).

Discussion

The non-invasive form of implantation in pigs results from endometrial secretion of protease inhibitors that regulate the highly invasive activity of the trophoblast (Roberts *et al.*, 1993). Therefore, although pig conceptuses are inherently highly proteolytic and invasive outside the uterine environment (Samuel and Perry, 1972), endometrial secretion of protease inhibitors only permits trophoblast attachment to the thick glycocalyx on the apical uterine surface epithelium (Stroband and Van der Lende, 1990). Placentation in pigs is associated with a reduction in the uterine epithelial glycocalyx as well as interdigitation of the trophoblastic and uterine microvilli (Danzter, 1985). Attachment of the trophoectoderm to the uterine surface glycocalyx can induce intracellular pathways that play a major role in pig placental attachment and embryo survival (Bowen *et al.*, 1996; Jaeger *et al.*, 2001).

Support for a principal function of the I α I family in matrix stabilization through its interaction with hyaluronic acid has been described for expansion of the

Fig. 1. Representative western blot analysis of protein (50 μ g) from (a) endometrial explant culture media and (b) uterine flushings on days of the oestrous cycle (C) and early pregnancy (P) in pigs using antiserum against human inter- α -trypsin inhibitor. Western blots were performed in triplicate to analyse the three animals per day in each status group. S: pig serum; Std: molecular mass standard.

cumulus-oocyte complex (Chen *et al.*, 1994). Identification of endometrial ITIH4 (Geisert *et al.*, 1998) and bikunin (Hettinger *et al.*, 2001) indicated that the I α I family is present in the pig uterus. The current study clearly establishes presence of the I α I family in the uterus and provides evidence that I α I may function to stabilize the glycocalyx at the endometrial-placental interface during pregnancy in pigs. The release of bikunin during early pregnancy (Hettinger *et al.*, 2001) may function to permit ITIH binding with hyaluronic acid and through its release in the interface between the conceptus and uterine epithelium prevent uterine invasion by the proteolytic conceptuses during placental attachment. Inactivation of the bikunin gene causes infertility in mice through disruption of the cumulus oophorus (Zhuo *et al.*, 2001) and this finding indicates that the breakdown in the pig uterine glycocalyx and embryonic loss that occurs after early (days 9–10) exposure to oestrogen (Blair *et al.*, 1991) may result from altered I α I production during

early stages of placentation. During the critical period of placental attachment in pigs on days 15–18 of pregnancy there is a 70-fold increase in endometrial bikunin gene expression (Hettinger *et al.*, 2001), which indicates that there is an important role for bikunin and the $\alpha 1$ heavy chains during the period of conceptus attachment to the uterine luminal surface.

Detection of immunoproteins of >200 and 140–120 kDa in endometrial culture medium by western blot analysis indicates that $\alpha 1$, P $\alpha 1$ and $\alpha 1$ L during pregnancy are present in the uterine endometrium of pigs. The two bands of approximately 140 and 120 kDa, which are present in pig serum, are consistent with the size of $\alpha 1$ L and P $\alpha 1$ (Rouet *et al.*, 1992). However, ITIH4 also has M_r of 120 kDa that would also be detected by the $\alpha 1$ antibody used in the study. Immunoreactive bands of M_r similar to $\alpha 1$, P $\alpha 1$ and $\alpha 1$ L were also detected in endometrial culture medium using antiserum to human bikunin (Hettinger *et al.*, 2001). These results indicate that the immunoproteins detected with $\alpha 1$ antibody represent bikunin bound with ITIH. It is possible that plasma $\alpha 1$ could contribute to the detection of uterine $\alpha 1$. However, the results of the present study demonstrate endometrial gene expression of all ITIH during the oestrous cycle and early pregnancy.

Although endometrial gene expression for ITIH was not different across the days of the oestrous cycle and early pregnancy, a distinct change in the release of the $\alpha 1$ family members was evident with *in vitro* culture of endometrium from cyclic and pregnant gilts. Detection of $\alpha 1$, $\alpha 1$ L and P $\alpha 1$ was greatly reduced in endometrial culture medium from pregnant gilts after day 12 of pregnancy. These results indicate that during attachment, the conceptuses either stabilize the release of the glycoproteins from the endometrial surface or proteolytic enzymes released by the conceptuses cleave $\alpha 1$, P $\alpha 1$ and $\alpha 1$ L. The alteration of the glycoproteins in culture medium was also observed in uterine flushings where detection of $\alpha 1$, P $\alpha 1$ and $\alpha 1$ L was reduced after day 10 of pregnancy. The presence of immunoproteins of lower M_r in uterine flushings of pregnant gilts indicates conceptus-induced proteolytic cleavage of ITIH. Cleavage of ITIH4 occurs specifically through activation of kallikrein that occurs *in vivo* after day 10 of the oestrous cycle or pregnancy (Geisert *et al.*, 1998; Vonnahme *et al.*, 1999). ITIH4 is present on the uterine surface and glandular epithelium and is detected in endometrial culture medium (Geisert *et al.*, 1995, 1998). Because ITIH4 does not contain protease inhibitory activity or bind bikunin like the other ITIH chains (Choi-Miura *et al.*, 1995; Nishimura *et al.*, 1995), ITIH4 must have a biological function unrelated to release of bikunin and protease inhibition. All ITIH contain a von Willebrand type-A domain that functions as a target for adhesion molecules (Salier *et al.*, 1996). Endometrial synthesis of ITIH4 could assist in the extracellular matrix formation through binding to integrins, collagen, proteoglycans and heparin. Choi-

Miura *et al.* (2000) proposed that ITIH4 binds and suppresses the phagocytic activity of polymorphonuclear cells. Thus, ITIH4 may serve an immunological protective role within the pig uterus.

The importance of the alteration in uterine $\alpha 1$, P $\alpha 1$ and $\alpha 1$ L during pregnancy may reflect the release of bikunin during association of heavy chains with hyaluronate (Jessen *et al.*, 1994). Although intact $\alpha 1$ (with bikunin) is able to bind to hyaluronate, bikunin is not associated with ITIH when bound to hyaluronate in extracellular physiological fluids (Jessen *et al.*, 1994). Bost *et al.* (1998) suggested that there is first an ionic interaction of hyaluronic acid with $\alpha 1$, which is followed by a substitution of hyaluronic acid for bikunin. The substitution would result in a complex consisting of heavy chains with only hyaluronic acid and the release of bikunin into extracellular fluids. The results of the present study indicate that such an alteration occurs during establishment of pregnancy and placentation in pigs. Hettinger *et al.* (2001) documented a pregnancy-specific alteration of bikunin release during *in vitro* endometrial culture similar to the present results. Furthermore, specific *in vitro* release of bikunin was only detected between day 15 and day 18 of pregnancy. Jessen *et al.* (1994) indicated that hyaluronate-bound $\alpha 1$ can be partly degraded *in vivo* by trypsin-like proteases, such as plasminogen activator. Presence of low M_r glycoproteins in the uterine flushings of pregnant gilts between day 10 and day 18 in the present study and the large increase in conceptus plasminogen activator release during placental attachment (Fazleabas *et al.*, 1983) provide evidence for cleavage of $\alpha 1$ during placental attachment in pigs. The combination of the results of the present study and other studies with the $\alpha 1$ family indicates the $\alpha 1$ family may have a physiological role in establishment of pregnancy in pigs.

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